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14. ABSTRACT

In Year 1 we examined the low concentration end of the full factorial design for our hibernation-based therapy for hemorrhagic shock (Specific Aim 1). We determined that high concentrations of the D-stereoisomer of beta-hydroxybutyrate (D-BHB) are required to improve survival. We also found that the other key component, melatonin, showed therapeutic benefits at concentrations that were 10-fold lower than previously published (Klein *et al.* 2010). In Year 2 we designed a dose-ranging study to determine if melatonin concentrations could be lowered further and still provide favorable outcomes. Survival curves were compared at 10 days following shock (60% blood loss for 1 hour). Treatments including both BHB and melatonin, even at melatonin concentrations 10^{-6} lower than previously published, were not statistically different ($p > 0.05$) from sham-operated animals with no blood loss. Shocked animals that received BHB only, or NaCl with melatonin, survived for statistically shorter times ($p < 0.05$) than shams. Consistent with Specific Aim 2, we also conducted a semi-log dose range from 0 to 50 mg/kg of 3-iodothyronamine in normotensive animals to corroborate that this thyroid hormone derivative possessed hypothermia-inducing properties. Temperature curves did not differ between treatments ($p > 0.05$). It is possible that hypothermic effects, if any, are masked by anesthesia. Experiments described in Specific Aim 3 have started, but this work is too preliminary to report in this document.

15. SUBJECT TERMS
hemorrhagic shock, blood loss therapy, D-beta-hydroxybutyrate, melatonin

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Introduction

In our Year 1 Annual Report, we reported experiments described in Specific Aim 1. These included a dose ranging study of BHB/M components D-stereoisomer beta-hydroxybutyrate (BHB), melatonin, and dimethyl sulfoxide (DMSO) in hemorrhagically shocked rats. This year, we have brought Specific Aim 1 close to completion. Also, we initiated experiments for Specific Aim 2 to determine if outcomes from hemorrhagic shock in rats can be improved by combining BHB/M with the proven hypothermia-promoting adjunct 3-iodothyronamine (T1AM); and Specific Aim 3 to determine the feasibility of administering a larger volume of a lower molarity BHB/M to hemorrhagically shocked rats. Results from these experiments will be described here.

Body

Specific Aim 1: Dose ranging study of BHB/M components D-Beta-Hydroxybutyrate, Melatonin, and DMSO in hemorrhagically shocked rats

Experimental Design

Since the results we reported in the Year 1 Annual Report provided evidence that melatonin provides beneficial effects at low concentrations, we reassessed the full-factorial design described in our Statement of Work and, after fixing the concentration of BHB at 4 M, we designed a melatonin dose-ranging study (Table 1). Also, we included sham-operated animals and NaCl controls. The surgical protocol was as previously described (Figure 1)

Survival

Survival curves were compared after 10 days following 60% blood loss (Figure 2) in SigmaPlot for Windows (version 11.0 Build 11.0.0.77) using a Logrank test. P-values for all comparisons are summarized in Table 2. Only two statistical differences were observed: 1) between sham-operated animals and the treatment containing 4 M BHB and 0 mM melatonin in 2% DMSO and 2) between sham-operated animals and the treatment containing 4 M NaCl and 0.000043 mM melatonin in 2% DMSO.

In the previous year, we suggested that “the therapeutic effects of melatonin… can still be observed at low concentrations. This is a reasonable assumption since serum melatonin peaks in rats are ~8.61×10^{-7} mM [1].” In our experimental procedure, the infused concentrations are diluted one thousand fold throughout the animal as they are administered at 1 ml/kg. That means that a 4.3 mM infusion will result in 4.3 x 10^{-3} plasma concentration; a 0.000043 mM infusion will in turn yield 4.3 x 10^{-8} mM in plasma. The data shown here provides evidence that melatonin can be lowered a million fold from the previously published concentration [2] and still provide survival benefits. Furthermore, we also confirm that melatonin is required for sustained survival.

Blood Gas Data

Blood samples were collected throughout the surgical protocol (refer to Figure 1) and analyzed in a blood gas analyzer (BGA) ABL815 Flex (Radiometer America). One-way ANOVAs with Tukey's *post hoc* test were performed to find treatment differences within different time points for total hemoglobin (tHb), pH, pressure of carbon dioxide (pCO_2), pressure of oxygen (pO_2), saturation of oxygen (sO_2), potassium ion (K^+), sodium ion (Na^+), calcium ion (Ca^{++}), chloride ion (Cl^-), glucose (Glu), and lactate (Lac).

Consistently, the Sham treatment showed statistical differences in all parameters, except for pH, at every time point after 1 ml/kg Infusion (refer to Figure 1). This is to be expected since Shams are neither hemorrhaged nor infused.

Although some other differences were observed, they can be attributed to individual variation and/or experiment-wide statistical error. All statistically significant observations are summarized in Table 3. We are currently in the process of assessing the feasibility of applying a multiple comparisons test other than Tukey's (e.g. Bonferroni, False Discovery Rate) in order to identify true differences.

Correlation to Survival

Regression analyses were performed in JMP 8.0 to elucidate whether survival could be predicted by any of the parameters measured by the BGA. We found no strong correlation between survival and tHb, Htc, pCO_2, pO_2, sO_2, K^+, Na^+, Ca^{++}, Cl^-, Glu or Lac sat any time point.

PowerLab Data

Physiological parameters such as mean arterial blood pressure (MAP), heart rate, and temperature were monitored during the whole procedure using a PowerLab 30/4 (ADInstruments). One-way ANOVAs with Tukey's *post hoc* test were performed to find treatment differences within time points. No correlation was observed for any of the parameters at any time point.

Correlation to Survival

As with BGA data, correlation tests between PowerLab data and survival were performed with the objective of clarifying whether MAP, heart rate, or temperature could forecast survival. No correlation was observed between survival and MAP, heart rate, or temperature at any time point.

Histology

Histopathological analyses are important for our research because in trauma, death is mostly encountered at three points: 1) within the first hour, 2) within the next 24 hours, 3) after days or weeks [3]. Since out post-operatory monitoring is not comprised of weeks, observing microanatomical changes in different tissues will provide information regarding the health status of the experimental subjects that survived the entire 10 days. It will also help us identify whether those animals would have kept living indefinitely or would have been likely to suffer health consequences in the near future.

Although a number of organs and systems can fail after trauma, we have decided to look at brain, intestine, and lung. The reasons are the following.

- Brain: the central nervous system remains the major single organ involved, not only in subsistence, but also quality of life. [3].
- Intestine: gut-derived factors contribute to a constellation of tissue, organ, and cellular responses that significantly contribute to the development of systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and multiple organ failure (MOF) [3, 4].
- Lung: the development of ARDS is a common step previous to the development of MOF in patients sustaining major trauma with significant blood loss [3].

A scoring system from 0 to 3 has been developed for each tissue and is summarized in Table 4.

Specific Aim 2: Determine if outcomes from hemorrhagic shock in rats can be improved by combining BHB/M with the proven hypothermia-promoting adjunct 3-Iodothyronamine (T1AM)

3-iodothyronamine (T1AM) has been shown to induce non-shivering hypothermia in mice [5]. For that reason, we hypothesized that T1AM, in combination with the optimized BHB/M can induce a hibernation-like protective state that improves recovery from normally lethal hemorrhagic shock.

Initially, we decided to investigate whether the observations made by Scanlan *et al* in non-anesthetized mice [5] could be replicated in a rat model. Therefore, we conducted a pilot study to determine whether T1AM could induce hypothermia in normotensive animals subjected to the instrumentation procedures common to our hemorrhagic shock model. The treatments included a semi-log dose range from 0 to 50 mg/kg in ten rats (n=2 per treatment). Though a reduction in temperature was observed, there were no observable differences between treatments (Figure 3). It is possible that the anesthesia could be masking the thyroid hormone derivative. It has been described that mice injected with T1AM take 6 to 8 hours to return to normal behavior [5]. However, we observed that rats recovered as soon as the anesthesia wore off (~10 minutes).

In the future, we will potentially return to Specific Aim 2 with experiments comparing the survival effect of adding T1AM to the optimized formulation. For now, since we did not observe any temperature effect, we will initiate Specific Aim 3.

Specific Aim 3: Determine the feasibility of administering a larger volume of a lower molarity BHB/M to hemorrhagically shocked rats

Currently, the standard of care, a medical or psychological treatment guideline, for hemorrhagic shock is based on studies performed between 1964 and 1975 by Shires [6, 7], Moyer [8], and Moss [9]. This guideline indicates the infusion of Lactated Ringer's Solution (LR) in three times the shed blood volume. If our resuscitation fluid is to be commercially and medically competitive, we need to perform studies comparing its efficacy to the standard of care.

The experimental procedures are summarized in Table 5 and Figure 4. Table 5 describes the treatments that will be administered. Figure 4 describes the surgical protocol.

Since Ketone Ringer's Solution (KR), a fluid similar to LR which substitutes lactate with BHB, has a 0.28 M concentration of BHB [10] and our hypertonic formulation contains a 4 M formulation of BHB, we will prepare our optimized BHB/M as in Specific Aim 1 and then dilute it 1:142 in distilled water. Table 6 provides a comparison of the composition of LR and diluted BHB/M.

We initiated these experiments on May 8th, 2013 and therefore do not have data to report at this time.

Key Research Accomplishments

- Completion of a melatonin dose-ranging study
- One million fold reduction in therapeutic melatonin concentration
- Initiation of histopathological analysis for Specific Aim 1
- Development of histopathological damage scoring system for brain, intestine, and lung.
- Initiation of experiments described in Specific Aim 2
- Initiation of experiments described in Specific Aim 3

Reportable Outcomes

In November 2012, the graduate student conducting the experiments, Cecilia Edna Pérez de Lara Rodríguez, presented the results from a portion of the melatonin dose-ranging study at the American Heart Association's Resuscitation Science Symposium which took place in Los Angeles, California. The abstract for this presentation was published in the journal Circulation [11] (see submitted abstract in the Appendices section, p. 23). In addition, Cecilia also gave a presentation at Hibernation 2.0 (January 2013, Oshkosh, Wisconsin). Her talk focused on the translation of the understanding of hibernation physiology into resuscitation science and the results obtained from the full melatonin dose-ranging study.

Conclusion

Specific Aim 1

- *Melatonin provides therapeutic effects at very low concentrations*. This is evident by the survival observed when administering a solution containing melatonin at a concentration 10^{-6}-fold lower than that published by Klein *et al* [12].
- *Melatonin is important for survival*. Rats administered 4M BHB without melatonin had the same ten-day survival as the 4M NaCl with .000043 mM melatonin controls.
- *BGA data did not provide information that helped elucidate treatment differences or predict survival. Neither did physiological data* such as MAP, heart rate, and temperature. However, monitoring of these parameters is of great importance throughout the surgical protocol (e.g. MAP needs to be observed for the endpoint of the first hemorrhagic period) and will continue to be monitored.

Specific Aim 2

- *Administration of T1AM did not influence temperature more than anesthesia*. It is possible that the latter may be masking the effects of the former because the mice of Scanlan *et al.* [5] were not anesthetized.

So What?

The surgical experiments for Specific Aim 1 have been concluded. Survival, BGA, and PowerLab data have been analyzed and are presented in this document. Histopathological analyzes have started but are not completed yet. In the Year 1 Annual Report we provided evidence that survival after 60% blood loss in our animal model for hemorrhagic shock is dose-dependent on the concentration of BHB infused, and for that reason we decided to fix it at 4 M. This year, we have provided evidence that melatonin can contribute favorably to survival even at concentrations one million fold lower than those previously published. We have decided not to explore infusing melatonin at any lower concentrations because, since any further dilution would fail to raise melatonin levels above endogenous concentrations, we do not believe it would provide any therapeutic benefit. We might, however, explore one more treatment: BHB/M without DMSO. There are three reasons for this: 1) the concentration of melatonin with which we are working now is water soluble, 2) DMSO acts solely as a solvent and not a therapeutic agent [12], and 3) DMSO has a bad reputation due to bad press in the 1960s [13] and there is much controversy over its use.

The observations of Scanlan *et al* [5] in mice did not translate into our anesthetized rat model. This suggests that allowing hemorrhaged individuals to passively cool may be sufficient hypothermia to provide a therapeutic effect and that a pharmacological induction of hypothermia may not affect survival. Due to this finding, we are considering the termination of the subcontract with Oregon Health and Sciences University for T1AM analyses.

Since the current standard of care for hemorrhagic shock cases is the administration of isotonic resuscitation fluids, Specific Aim 3 describes experiments where the optimized BHB/M is administered at isotonic concentrations. We have started these experiments and will have data to report in our next Quarterly Report.

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13. Carley, W., *DMSO may have caused death of woman, makers of 'Wonder' drug warn doctors*, in *Wall Street Journal*1965: New York City.

Appendices

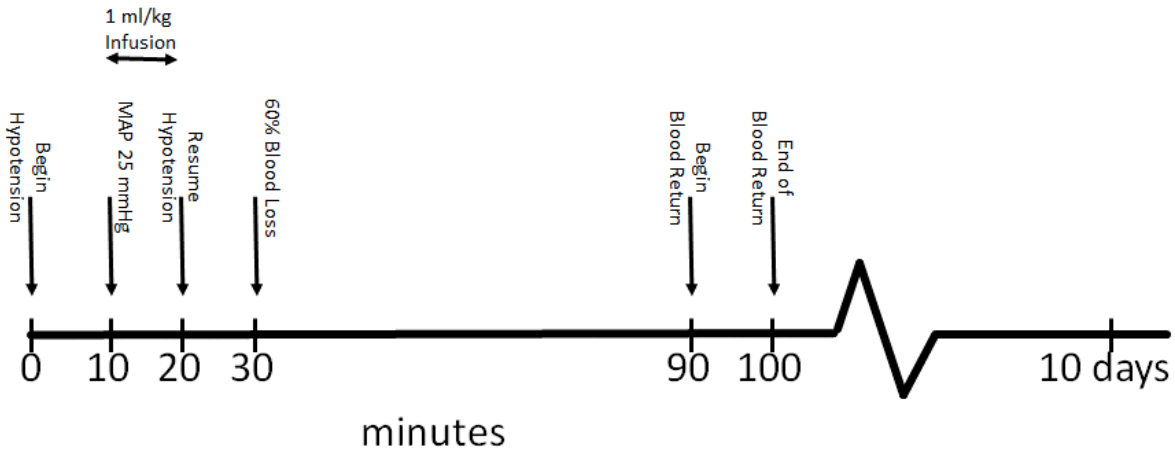
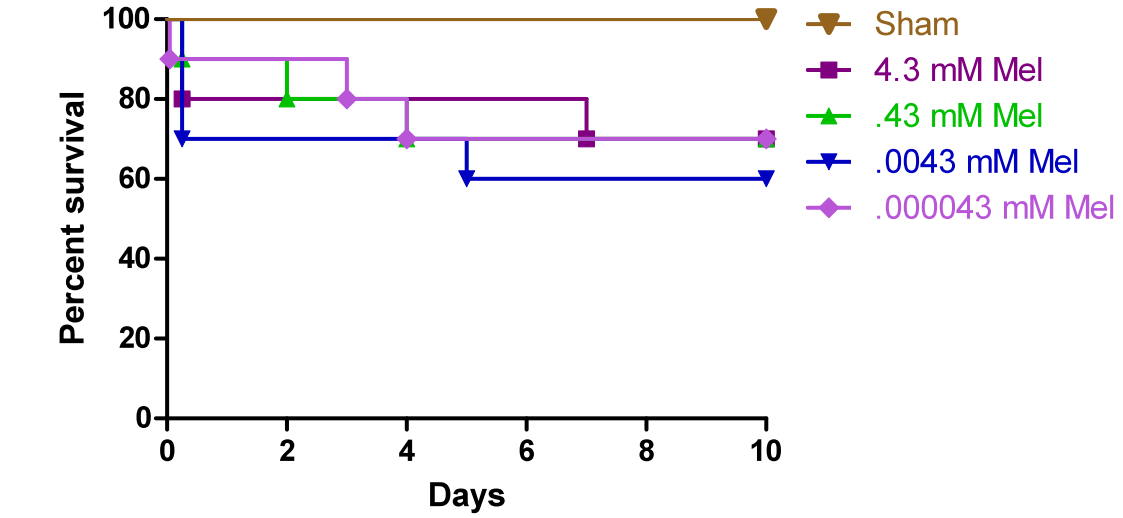


Figure 1. Timeline of surgical protocol for Specific Aims 1 and 2. After surgical instrumentations, rats are hemorrhaged to 25 mmHg over a ten minute period. Then, they are infused with the different treatments at a dose of 1 mg/kg over ten minutes. The animals are further hemorrhaged for an additional ten minutes to remove a total of 60% of its calculated blood volume. Animals are kept in shock for an hour before being given a blood transfusion equal to 50% of the total blood withdrawn during the hemorrhagic period at a rate of 500 µl/minute. After having their wounds closed, survival is monitored for ten days.



A

B

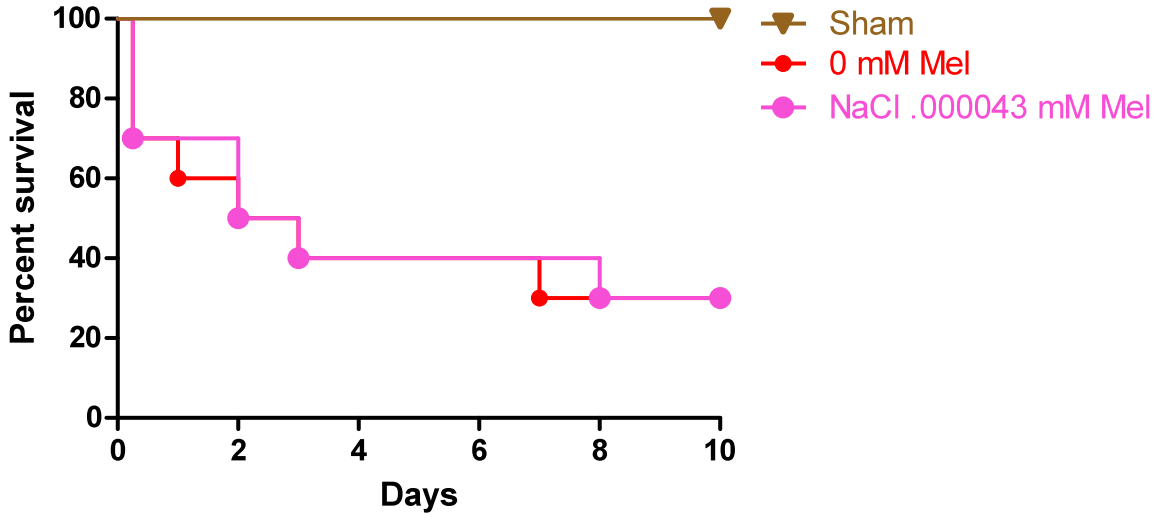


Figure 2. Kaplan-Meier survival plots of melatonin dose-ranging study. A. Treatments not statistically different ($p > 0.05$) to Shams. B. Treatments statistically different ($p < 0.05$) to Shams. All treatments labeled only as melatonin (Mel) contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

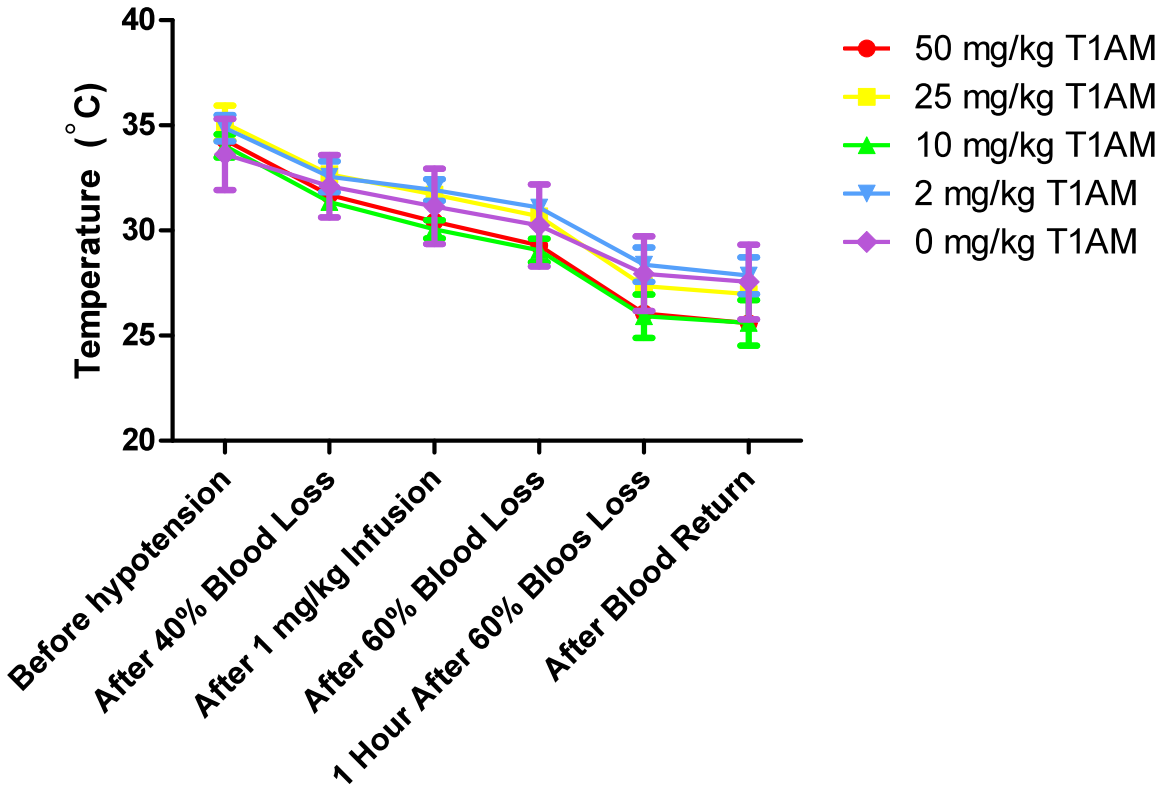
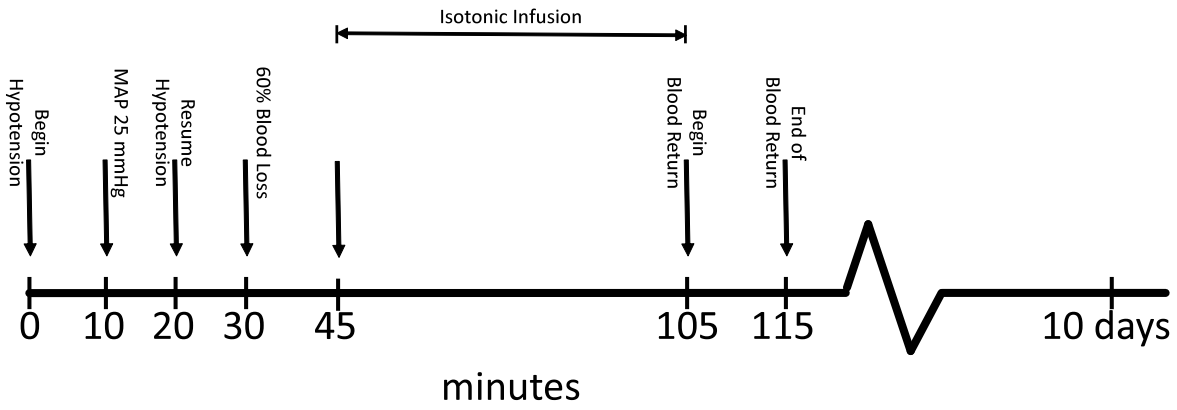


Figure 3. Rectal temperature over time for T1AM pilot study. The X-axis represent hallmarks of the surgical protocol represented in Figure 1. These animals, however, were not hemorrhaged or received a blood transfusion. All treatments contain 2% DMSO. N= 2 for all treatments.

A



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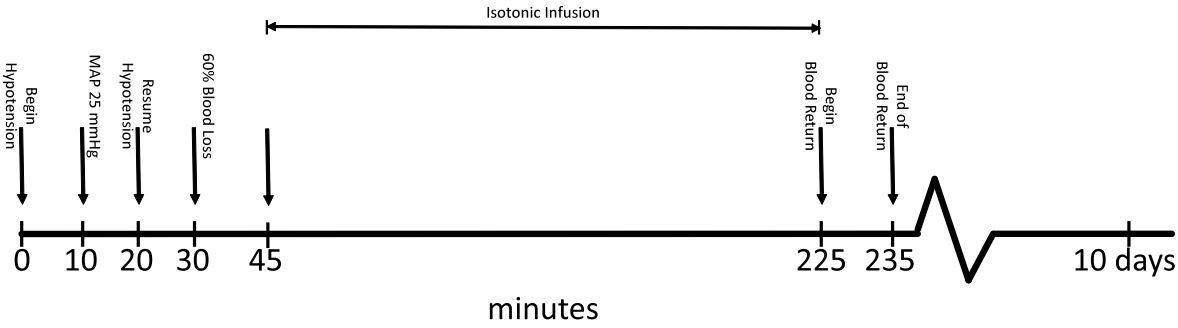

Figure 4. Timeline of surgical protocol for Specific Aim 3. After surgical instrumentations, rats are hemorrhaged to 25 mmHg over a ten minute period. No blood is withdrawn nor is infusion administered for ten minutes. Then, another ten minutes are employed to further hemorrhage the animal to 60% of its calculated blood volume. Animals are infused for either an hour (A) or three hours (B) at a rate of one times the blood volume withdrawn per hour before being given a blood transfusion equal to 50% of the total blood withdrawn during the hemorrhagic period at a rate of 500 µl/minute. After having their wounds closed, their survival is monitored for ten days.

Table 1. Melatonin dose-ranging study design.

BHB (M)	Mel (mM)	DMSO
4	4.3	2%
4	0.43	2%
4	0.0043	2%
4	0.000043	2%
4	None	2%

Table 2. P-values for melatonin dose-ranging study. Significant values are highlighted in light orange. All treatments labeled only as melatonin contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

Comparisons	P Value
0 mM Mel vs. Sham	0.00064
Sham vs. NaCl .000043 mM Mel	0.00066
0.000043 mM Mel vs. NaCl .000043 mM Mel	0.07910
0.0043 mM Mel vs. 0.000043 mM Me	0.68800
0.0043 mM Mel vs. NaCl .000043 mM Mel	0.22800
0.0043 mM Mel vs. Sham	0.02220
0.43 mM Mel vs. 0.000043 mM Mel	0.99800
0.43 mM Mel vs. 0.0043 mM Mel	0.63600
0.43 mM Mel vs. NaCl .000043 mM Mel	0.07700
0.43 mM Mel vs. Sham	0.05480
0 mM Mel vs. 0.000043 mM Mel	0.07670
0 mM Mel vs. 0.0043 mM Mel	0.22200
0 mM Mel vs. 0.43 mM Mel	0.07220
0 mM Mel vs. 4.3 mM Mel	0.07960
0 mM Mel vs. NaCl .000043 mM Mel	0.91300
4.3 mM Mel vs. 0.000043 mM Mel	0.96300
4.3 mM Mel vs. 0.0043 mM Mel	0.61900
4.3 mM Mel vs. 0.43 mM Mel	0.99700
4.3 mM Mel vs. NaCl .000043 mM Mel	0.08930
4.3 mM Mel vs. Sham	0.05490
Sham vs. 0.000043 mM Mel	0.05480

Table 3. P-values for different BGA parameters. Only comparisons where at least one significant value was observed are presented. Significant values are highlighted in light orange. All treatments labeled only as melatonin contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

tHb					
	Before Hypotension	After 40% Blood Loss	After Bolus	After 60% Blood Loss	1 hour after 60%	After Blood Return
0.000043 mM Mel vs Sham	0.99913	0.42022	0.00000	0.00000	0.00013	0.94910
0.0043 mM Mel vs Sham	0.99867	0.00364	0.00000	0.00000	0.00000	0.13365
0.43 mM Mel vs Sham	1.00000	0.00015	0.00000	0.00000	0.00000	0.55641
No Mel vs Sham	0.91966	0.03172	0.00000	0.00000	0.00000	0.47138
4.3 mM Mel vs Sham	0.97703	0.05539	0.00000	0.00000	0.00018	0.66187
NaCl .000043 mM Mel vs Sham	0.78134	0.28537	0.00000	0.00000	0.00004	0.07252
pCO2						
	Before Hypotension	After 40% Blood Loss	After Bolus	After 60% Blood Loss	1 hour after 60%	After Blood Return
0.000043 mM Mel vs Sham	1.00000	0.02430	0.00048	0.00002	0.00000	1.00000
0.0043 mM Mel vs No Mel	0.02772	0.34048	0.01590	0.98471	0.99643	0.95288
0.0043 mM Mel vs Sham	0.77313	0.25876	0.19820	0.00001	0.00000	1.00000
0.43 mM Mel vs Sham	1.00000	0.00082	0.00529	0.00004	0.00000	0.99994
No Mel vs Sham	0.74932	0.00026	0.00000	0.00000	0.00000	0.90350
4.3 mM Mel vs Sham	0.99266	0.05289	0.05512	0.00086	0.00004	1.00000
NaCl .000043 mM Mel vs Sham	0.66948	0.69842	0.03701	0.00086	0.00000	0.99875
pO2						
	Before Hypotension	After 40% Blood Loss	After Bolus	After 60% Blood Loss	1 hour after 60%	After Blood Return
0.000043 mM Mel vs Sham	1.00000	0.13833	0.08653	0.04571	0.00044	1.00000
0.0043 mM Mel vs Sham	0.87834	0.00204	0.89724	0.01163	0.00006	0.65645
0.43 mM Mel vs Sham	0.99976	0.00063	0.62961	0.81947	0.00057	0.99549
No Mel vs Sham	1.00000	0.00037	0.06717	0.01289	0.00001	0.88764
4.3 mM Mel vs Sham	1.00000	0.00126	0.55596	0.03579	0.00072	0.99995

Table 3 continued. P-values for different BGA parameters. Only comparisons where at least one significant value was observed are presented. Significant values are highlighted in light orange. All treatments labeled only as melatonin contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

sO2						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs NaCl .000043 mM Mel	1.00000	0.61915	0.00191	0.91514	1.00000	1.00000
0.0043 mM Mel vs NaCl .000043 mM Mel	0.10438	0.05385	0.01794	1.00000	1.00000	0.92783
0.0043 mM Mel vs Sham	0.00600	1.00000	0.56576	0.99932	1.00000	0.61513
0.43 mM Mel vs NaCl .000043 mM Mel	0.99901	0.09675	0.01998	0.99997	0.99125	1.00000
No Mel vs NaCl .000043 mM Mel	0.99811	0.06813	0.00106	0.99135	0.98682	0.99761
4.3 mM Mel vs NaCl .000043 mM Mel	0.97089	0.10409	0.00627	0.99982	0.99857	0.98759
K+						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs Sham	1.00000	0.00000	0.58490	0.69613	0.00696	0.00169
0.0043 mM Mel vs 0.43 mM Mel	0.67100	0.21867	0.18053	0.03386	0.05443	0.11111
0.0043 mM Mel vs NaCl .000043 mM Mel	0.01574	0.25938	0.99672	0.98414	0.99762	0.97539
0.0043 mM Mel vs Sham	0.95409	0.00000	0.00227	0.00048	0.55664	0.06561
0.43 mM Mel vs Sham	0.99966	0.00016	0.92250	0.99633	0.00002	0.00001
No Mel vs Sham	1.00000	0.00002	0.16090	0.26650	0.00022	0.00001
4.3 mM Mel vs Sham	0.98511	0.00000	0.88528	0.87331	0.00449	0.00103
NaCl .000043 mM Mel vs Sham	0.32449	0.00002	0.03675	0.03965	0.10903	0.00312

Table 3 continued. P-values for different BGA parameters. Only comparisons where at least one significant value was observed are presented. Significant values are highlighted in light orange. All treatments labeled only as melatonin contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

Na+						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs 0.43 mM Mel	0.06931	0.01976	0.00099	0.09378	0.09442	0.39159
0.000043 mM Mel vs 4.3 mM Mel	0.83675	0.99736	0.99903	0.99720	0.96502	0.99776
0.000043 mM Mel vs NaCl .000043 mM Mel	0.01378	0.06913	0.85537	0.58991	0.15650	0.40332
0.000043 mM Mel vs Sham	0.98667	0.09106	0.47612	0.95355	0.05658	0.01858
0.0043 mM Mel vs NaCl .000043 mM Mel	0.01624	0.32511	1.00000	0.99845	0.99982	0.99987
0.0043 mM Mel vs Sham	0.99508	0.00332	0.99991	0.13401	0.99473	0.42000
0.43 mM Mel vs 4.3 mM Mel	0.00020	0.00180	0.00021	0.00789	0.71383	0.84740
0.43 mM Mel vs Sham	0.46017	0.00000	0.29339	0.00082	1.00000	0.85580
No Mel vs Sham	1.00000	0.00407	1.00000	0.09623	1.00000	0.31917
4.3 mM Mel vs NaCl .000043 mM Mel	0.00002	0.00698	0.44374	0.12203	0.83997	0.86223
NaCl .000043 mM Mel vs Sham	0.15929	0.00000	0.99972	0.02410	1.00000	0.80073
Ca++						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs NaCl .000043 mM Mel	0.02497	0.17375	0.53439	0.99345	0.99043	1.00000
0.000043 mM Mel vs Sham	0.89359	0.00002	0.04790	0.99999	0.50080	0.99960
0.0043 mM Mel vs NaCl .000043 mM Mel	0.02609	0.61446	1.00000	1.00000	0.99973	1.00000
0.0043 mM Mel vs Sham	0.92168	0.00027	0.92508	0.92426	0.73792	1.00000
0.43 mM Mel vs Sham	0.97746	0.57039	0.00154	0.99993	0.40723	0.51733
No Mel vs NaCl .000043 mM Mel	0.00899	0.82516	0.62790	0.95510	0.99992	1.00000
No Mel vs Sham	0.79487	0.00183	0.05715	1.00000	0.78957	0.99994
4.3 mM Mel vs NaCl .000043 mM Mel	0.04785	0.56712	1.00000	1.00000	0.99947	0.99760
4.3 mM Mel vs Sham	0.97136	0.00059	0.91519	0.98835	0.70111	0.93902

Table 3 continued. P-values for different BGA parameters. Only comparisons where at least one significant value was observed are presented. Significant values are highlighted in light orange. All treatments labeled only as melatonin contain 4 M BHB. NaCl treatment is also 4 M. All treatments contain 2% DMSO. N= 10 for all treatments.

Cl-						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs NaCl .000043 mM Mel	0.58625	0.50369	0.00978	0.28055	0.01695	0.99815
0.0043 mM Mel vs NaCl .000043 mM Mel	0.16526	0.10310	0.00000	0.00198	0.00010	0.09938
0.0043 mM Mel vs Sham	0.46075	0.03412	0.81263	0.01195	0.08581	0.02406
0.43 mM Mel vs NaCl .000043 mM Mel	0.29243	0.45937	0.00001	0.00284	0.00001	0.07047
0.43 mM Mel vs Sham	0.65331	0.24263	0.63494	0.01482	0.01113	0.01847
No Mel vs NaCl .000043 mM Mel	0.11372	0.72535	0.00002	0.01232	0.00003	0.15414
No Mel vs Sham	0.36656	0.47940	0.95981	0.05727	0.02922	0.04272
4.3 mM Mel vs NaCl .000043 mM Mel	0.58015	0.97040	0.00051	0.03670	0.00013	0.35069
NaCl .000043 mM Mel vs Sham	0.99989	1.00000	0.00215	0.99977	0.40141	0.99984
Glu						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs No Mel	0.99068	0.98240	0.12836	0.00433	0.01809	0.58670
0.000043 mM Mel vs 4.3 mM Mel	0.99964	0.97870	0.78399	0.10732	0.03892	0.73284
0.000043 mM Mel vs NaCl .000043 mM Mel	0.95869	0.65744	0.04415	0.00191	0.08187	0.34092
0.000043 mM Mel vs Sham	0.99999	0.64810	0.02462	0.24381	0.00923	0.01846
0.0043 mM Mel vs Sham	0.99961	0.74730	0.00080	0.00158	0.00022	0.00000
0.43 mM Mel vs Sham	0.59537	0.30544	0.00003	0.00001	0.00000	0.00000
No Mel vs Sham	0.99979	0.07097	0.00000	0.00000	0.00000	0.00000
4.3 mM Mel vs Sham	1.00000	0.10136	0.00013	0.00001	0.00000	0.00000
NaCl .000043 mM Mel vs Sham	0.99644	0.00335	0.00000	0.00000	0.00000	0.00000
Lac						
	Before Hypotension	**After 40% Blood Loss**	**After Bolus**	**After 60% Blood Loss**	**1 hour after 60%**	**After Blood Return**
0.000043 mM Mel vs Sham	1.00000	0.00570	0.00000	0.00000	0.00020	0.01737
0.0043 mM Mel vs Sham	0.99796	0.01991	0.00000	0.00000	0.00003	0.00005
0.43 mM Mel vs Sham	0.86986	0.00003	0.00000	0.00000	0.00361	0.05367
No Mel vs NaCl .000043 mM Mel	0.99985	0.99940		0.03291	0.54905	0.68240
No Mel vs Sham	0.99845	0.00096	0.00000	0.00000	0.00000	0.00023
4.3 mM Mel vs Sham	0.99811	0.02180	0.00000	0.00000	0.00005	0.00629
NaCl .000043 mM Mel vs Sham	1.00000	0.00002	0.00000	0.00000	0.01203	0.05042

Table 4. Histopathological scoring system.

		Lung	Intestine	Brain
0		No Evidence		
1	Mild	Alveolitis (2-3X), Perivascular edema	Development of subepithelial Gruenhagen's space, vacuolization at the villus tip	Focal pyknosis
2	Moderate	Alveolitis (3-4X), Interstitial edema	Lifting of epithelial layer from the lamina propria, Increased vacuolization from the tip to midportion of villi,	Multifocal pyknosis
3	Severe	Alveolitis (>5X), Alveolar edema, Inflammatory infiltrate, Hemorrhage	Epithelial lifting and vacuolization from the tip to lower portion of villi, Mucosal ulceration and disintegration of the lamina propria, Inflammatory infiltrate, Hemorrhage	Extensive pyknosis

Table 5. Isotonic formulation study design.

60 min Shock	180 min Shock
Dilute BHB/M	
Lactated Ringer	
Shams	

Table 6. Components of Lactated Ringer's and Dilute BHB/M Solutions.

Component	Lactated Ringer's	Dilute BHB/M
3-D-β-hydroxybutyrate		28 mM
D-L-lactate	28 mM	
Sodium	130 mM	28 mM
Potassium	4 mM	
Calcium	3 mM	
Chloride	109 mM	
Melatonin		$3x10^{-7}$ mM
DMSO		0.015%

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timization of a Hibernation Based Small Volume Resuscitation Fluid

thor Block: Cecilia E Perez de Lara Rodriguez, Alison Kingsbury, Lester R Drewes, Matthew T Andrews, Univ of Minnesota Duluth, Duluth, MN

stract:

ckground: Traumatic injury is the main cause of death among individuals ages 1 to 44 in the US. Hibernation depicts a notable physiological state featuring low cardiac output and blood w similar in degree to hemorrhagic shock, yet the brain and other tissues of hibernators are naturally shielded from ischemia and reperfusion injury during arousal from torpor. A small volume suscitation fluid based on hibernation physiology has been developed in our laboratory. The current formula of 4M D β-hydroxybutyrate (BHB), 43mM melatonin, and 20% DMSO has proven ective in extending survival in rat and pig models of hemorrhagic shock. The research described here intends to optimize the therapeutic concentrations of BHB, melatonin, and dimethyl foxide (DMSO) as well as the delivery of this therapy.

ethods and Results: Following surgical preparation, rats underwent hemorrhagic shock by the controlled withdrawal of 60% of their blood volume. Previously, our therapy had been infused in o phases: 1) a bolus of 1 ml/kg over a ten minute period and 2) a slow infusion of 100 µl/hr over one hour. Acute (no blood return) surgeries were performed on groups receiving either 1) a lus plus slow infusion or 2) the bolus only. No statistical difference ($p>0.05$) was observed. Subsequently, blood return surgeries were performed. Dose ranging studies of BHB and melatonin re done independently. BHB concentrations used were 4M, 2M, and 0.4 M. No significant difference ($p>0.05$) was observed. However, a dose dependent trend was noted. The dose ranging udy of melatonin included concentrations of 43mM, 4.3mM, and 0mM. All treatments had 4M BHB. There was no statistical difference ($p>0.05$) in survival between 43mM and 4.3mM latonin. At 4.3mM, melatonin was statistically ($p<0.05$) more efficient at achieving 10 day survival compared to 0mM melatonin.

nclusions: The infusion of a 1 ml/kg bolus of our small volume resuscitation fluid is just as effective in expanding the "golden hour" as the continuation of the infusion at a slow rate. BHB has dose dependent effect on survival and for that reason we have decided to fix the concentration of BHB for all further formulations at 4M. Melatonin enhances survival, yet its therapeutic effects n still be observed at low concentrations.

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